FABRICATION OF NANOPARTICLES TO TREAT METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

An Undergraduate Research Scholars Thesis

by

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Submitted to the Undergraduate Research Scholars program
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Carolyn Cannon

May 2016

Major: Biochemistry

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ABSTRACT

Fabrication of Nanoparticles to treat Methicillin-resistant Staphylococcus aureus

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Staphylococcus aureus (SA) lung infections are a major problem for patients with cystic fibrous (CF). As these infections are difficult to treat, they can be life-threatening.[1] Moreover, methicillin-resistant *Staphylococcus aureus* (MRSA) strains have become more prevalent and often exhibit resistance to first line antibiotics, including vancomycin, clindamycin, and trimethoprim/sulfamethoxazole.[2] A natural product, MC21-A (C58), from a marine bacteria, *Pseudoalteromonas phenolica*, has potent activity against MRSA. However, the compound is hydrophobic; thus, delivery in an aqueous solution is problematic.[3] In order to optimize the efficacy of this drug candidate to treat MRSA lung infections, it must pass through the thick mucus in the CF airway to reach the bacteria. Encapsulating C58 in a polymeric nanoparticle can improve penetration into and retention in mucus and bacterial biofilms. In particular, nanoparticles coated with polyethylene glycol (PEG) have been shown to rapidly penetrate human mucus.[4] We have developed a simple, scalable method for production of poly (lactic-co-glycolic acid) (PLGA) nanoparticles coated with PEG that allows large-scale production of this "workhorse" nanoparticle system. These nanoparticles will allow further testing of this drug and other potential candidates to proceed to clinical testing for treatment of MRSA lung infections in CF patients.

DEDICATION

I would like to dedicate this paper to my parents, Mary and Bernard Kinane, without whom I would not have been able to get to where I am today.

ACKNOWLEDGMENTS

I would like to thank Dr. Carolyn Cannon for her support at beginning this research and for her encouragement along the way. In addition I would like to thank Dr. Kush Shah, for teaching me all the required methods and for proof reading my paper many times, Dr. Parth Shah, for his constructive criticism and Kevin Chen, for helping in the editing process and for keeping my morale high. Finally, I would like to thank Ava Brozovich for her moral support.

NOMENCLATURE

CF- cystic fibrosis

PLGA – poly(lactic-co-glycolic acid)

PEG- polyethylene glycol

MRSA- methicillin-resistant *Staphylococcus aureus*

SA- *Staphylococcus aureus*

CHAPTER I

INTRODUCTION

Cystic fibrosis (CF) is the most common, fatal genetic disease among Caucasians in the United States, affecting an estimated 30,000 people. [1] It is inherited in an autosomal recessive pattern and affects multiple organs including the lungs, pancreas and gastrointestinal tract.[5] Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are responsible for this disease; the gene codes for the CFTR protein, which is a chloride channel. Patients with CF produce thick and sticky mucus that clogs the lungs. Mucus also blocks the pancreatic ducts, which stops digestive enzymes from reaching the intestine. This ion channel, CFTR, is expressed on the surface of the airway epithelial cells and is responsible for the transport of chloride ions into the airway in the non-CF population. The chloride ions are followed by sodium ions and water molecules follow the salt into the airway in order to maintain the ionic and osmotic balance; this migration of NaCl and water results in the lining of the airway by a hydrated mucous layer. In CF patients, CFTR is mutated and the chloride ion transport is reduced or absent. This reduced or absent chloride transport results in the absence of water transport leading to the development of thick and sticky mucus that lines the airway. The thick mucus is an ideal medium for bacteria to grow including *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Indeed, the lungs are chronically infected and have to be treated with multiple courses of antibiotics each year.[5,6] Accordingly, the bacteria become resistant to standard-of-care antibiotics and new antibiotics that can penetrate the airway mucus are warranted.

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) lung infections has escalated among cystic fibrosis patients in the past two decades.[2] Although outcomes of MRSA infection in CF patients can vary, MRSA has recently been shown to constitute an independent risk factor for mortality.[1] Treatment of these infections is complicated by the increasing antibiotic resistance.[2] Other antibiotics including linezolid and daptomycin have been developed to address the increasing prevalence of MRSA.[7] However, resistance to these new antibiotics has already been seen.[8] Vancomycin has been the treatment of choice for serious MRSA infections since 1958. However, its overuse has led to the emergence of vancomycin-intermediate and -resistant MRSA.[9] While the need for new antibiotics to treat MRSA is urgently needed, the rate of the Food and Drug Administration (FDA) approval of new antibiotics to address this need is declining. To further complicate the picture, the growth of MRSA in biofilms embedded in the thick, dehydrated mucus found in the airways of CF patients is problematic to treat due to inherent resistance of bacteria in biofilm growth mode, as well as difficulty in delivering high concentrations of antibiotics to the site of infection. For example, the gold-standard antibiotic for MRSA infections, vancomycin, has poor penetration into the lung, in general, and through mucus and biofilms, in particular. [10] Thus, new treatments for MRSA lung infections are needed.

Drug Development

A natural product, MC21-A (or C58), isolated from a marine bacterium, *Pseudoalteromonas phenolica* O-BC30(T), has potent activity against MRSA. This promising antimicrobial agent eradicates MRSA at a higher rate compared to vancomycin, without killing human cells.[3,11] Recent improvements in the production of C58 will allow for the generation of sufficient quantities in order for this drug to be used in clinical studies. *In vitro* studies by the Cannon

group have verified the antimicrobial activity of C58. Compared to vancomycin, C58 demonstrates superior activity against planktonic, as well as biofilm associated MRSA.

Nanoparticle and Drug Delivery

Using nanoparticles to deliver drugs is an intense area of research that has been successfully translated to the clinic. For example, in 2013, the FDA approved a paclitaxel albumin-stabilized nanoparticle formulation for the treatment of metastatic adenocarcinoma of the pancreas. This formulation prolongs the stability and the action of the chemotherapeutic agent, paclitaxel.[12] The approval of paclitaxel albumin-stabilized nanoparticle formulation for the treatment of this fatal cancer was based on the demonstration of improved overall survival in a clinical trial of patients with metastatic pancreatic cancer.

Nanoparticles can be delivered by different routes including intravenous and aerosol. Indeed, nanoparticles can deliver antibiotics to the lungs via either an intravenous or inhaled route.[10] The latter routes is more highly desired, as it limits the exposure of the other organs to the delivered medication.

Nanoparticles can be used to target drugs to specific locations in the body by altering the nanoparticles' surface qualities. Each nanoparticle system must be optimized to achieve the desired targeted delivery. To achieve bacterial killing of MRSA in the biofilms of the CF lung, C58 must penetrate the thick mucus in which the microorganisms are embedded. Airways mucus normally traps and removes foreign particles such as nanoparticles via mucociliary clearance.[13] Thus, it is necessary to design nanoparticles capable of rapid penetration through mucus in order to avoid swift clearance from the airway. Nanoparticles composed of poly(lactic-co-glycolic acid) and coated with polyethylene glycol (PEG) have been shown to rapidly penetrate human

mucus and are likely to evade removal from the airway by mucociliary clearance.[4] Thus, encapsulating C58 in PLGA-PEG nanoparticles should improve penetration into mucus and optimize the efficacy of this drug candidate to treat CF MRSA lung infections.

Nanoparticles can be generated by a number of techniques including nanoprecipitation and emulsion solvent evaporation. The nanoprecipitation technique has been shown to be superior for loading of nanoparticles with water insoluble drugs such as C58.[14] Recently, there has been further refinement of the nanoprecipitation method to generate particle size less than 150 nm with narrow distribution of size; these smaller particles are likely to evade mucociliary clearance.[15]

CHAPTER II

METHODS

Nanoparticles were fabricated using a nanoprecipitation technique. Parameters that affect nanoparticle size, shape, and morphology include the ratio of the polymer PLGA to the amphiphilic coating, poly(lactic-co-glycolic acid)-polyethylene glycol (PEG) and concentrations of solvent and non-solvents. Polymers PLGA and PEG were dissolved in acetonitrile of varying concentrations in combination with dimethyl sulfoxide (DMSO).[16] The polymer solution was added to the non-solvent solution consisting of a surfactant, polyvinylpyrrolidone (PVP). The addition of polymer was performed via a drop-wise method in order to enhance nanoparticle formation and prevent aggregation. The ratios of PLGA and PEG, as well as the concentrations and combinations of solvents and non-solvent solution were optimized to achieve the desired nanoparticle size, shape, and morphology. In addition, particle size and charge are critical factors for the transition of the particle through the airway mucus/sputum. In order to assess these particle parameters, the resultant nanoparticle suspension was then centrifuged and washed with de-ionized water three times to remove PVP and aggregates of polymer. Finally, the nanoparticles were freeze-dried to a constant weight.

Once the nanoparticle formulation was developed, the size and morphology of the nanoparticles were determined using scanning electron microscopy and dynamic light scattering. The particles were coated with silver-palladium using a sputter-coater and imaged with a scanning electron microscope. Images were utilized to analyze the size and shape of the nanoparticles. The size, the distribution of molecular mass (polydispersity index), and surface charge were determined using

dynamic light scattering on a Brookhaven ZetaPlus system. This is a non-invasive technique for measuring the size and size distribution of molecules in the micron and submicron region.

Optimized parameters that yield nanoparticles with desirable characteristics were then used to formulate C58 drug loaded nanoparticles. Briefly, C58 was dissolved with the polymer solution and added to the non-solvent phase as described earlier. The concentration of C58 was 1%, 2.5%, and 5% w/w to the polymer mass to yield C58 loaded PLGA nanoparticles. At the conclusion of these experiments, we identified the optimized, ideal conditions for generation of nanoparticles for delivery of antibiotics such as C58.

CHAPTER III

RESULTS

The PLGA-PEG was dissolved in various concentrations of acetonitrile. For some experiments, acetonitrile and DMSO were used in order to enhance the solubility of PLGA-PEG. Likewise PLGA-PEG was "dropped" into various concentration of the non-solvent PVP. If suspended nanoparticles could be generated, the experimental conditions were deemed appropriate and the results of the experiment would be designated "pass". If no suspendable particles could be generated, the experimental result was designated a "fail". Thus, for each experimental condition, the results were reported as a pass or fail. Under some conditions, if nanoparticles were generated, but could not be suspended despite two attempts to do so, the results of were reported as "fail/pass".

Table 1 PLGA in the absence of PEG

%Acetonitrile (solvent)	PLGA mg/mL	% PVP (non-solvent)	Result
80%	25	0%	**Fail**
80%	25	0.5%	**Fail**
80%	25	1%	**Fail**
95%	25	2%	**Fail**
95%	25	1%	**Fail**
80%	50	2%	**Fail**
80%	50	1%	**Fail**
95%	50	5%	**Fail**
95%	50	2%	**Fail**
95%	50	1%	**Fail**
95%	100	2%	**Fail**
95%	100	1%	**Fail**
100%	50	5%	**Fail**
100%	50	2%	**Fail**
100%	50	1%	**Fail**
100%	50	0.5%	**Fail**

For the first experimental condition, PLGA alone was used to determine if nanoparticles could be generated in the absence of PEG; this experiment would establish if PEG were necessary (Table 1). It was determined that PLGA alone (PEG free) did not generate nanoparticles using

nanoprecipitation no matter what concentrations of acetonitrile or PVP were used. It was concluded that PEG was needed in order to generate nanoparticles.

Initially, lower concentrations of PEG were used in order to be cost effective. Thus, experiments were performed using 0.5% PEG (Table 2). Likewise, the addition of 0.5% PEG did not result in the generation of nanoparticles. Higher concentrations of PEG were deemed necessary.

Table 2 – PLGA combined with 0.5% PEG.

% Acetonitrile (solvent)	**PLGA mg/mL**	**% PVP (non-solvent)**	**Result**
100%	50	2.5%	**Fail**
100%	50	2%	**Fail**
100%	50	1.5%	**Fail**

Further experiments were performed with 1% PEG (Table 3). Again, particles were not generated. Sequential experiments were performed by increasing the PEG concentration by 0.5% intervals until reaching an optimal concentration of 3% PEG.

Table 3 PLGA combined with 1% PEG.

% Acetonitrile (solvent)	**PLGA mg/mL**	**% PVP (non-solvent)**	**Result**
80%	25	2%	**Fail**
80%	25	1%	**Fail**
95%	25	2%	**Fail**
95%	25	1%	**Fail**
80%	50	2%	**Fail**
80%	50	1%	**Fail**
95%	50	5%	**Fail**
95%	50	2%	**Fail**
95%	50	1%	**Fail**
100%	50	2.5%	**Fail**
100%	50	2%	**Fail**

Ultimately, the concentration of PEG was increased to 3.5% and robust particle formation was observed. The optimal conditions for the generation of nanoparticles were: **PLGA (50 mg/mL) combined with 3% PEG that was dissolved in 80% acetonitrile, which was in turn, "dropped" into 1.5% or 2.5% PVP** (Table 4).

Table 4

PLGA combined with 2.5% PEG

% Acetonitrile (solvent)	PLGA mg/ mL	% PVP (non-solvent)	Result
100%	50	2.5%	Pass
100%	50	2%	Pass
100%	50	1.5%	Pass

PLGA combined with 3% PEG

% Acetonitrile (solvent)	PLGA mg/ mL	% PVP (non-solvent)	Result
80%	50	2.5%	~Pass
80%	50	2%	Pass*
80%	50	1.5%	Pass*

As the solvent is critical for particle formation, we explored whether or not other solvents may be used. Acetonitrile was substituted with acetone or acetone plus DMF. Acetonitrile was the only successful solvent (Table 5).

Table 5. PLGA combined with 3% PEG

% Acetone (solvent)	PLGA mg/ mL	% PVP (non-solvent)	Result
80%	50	2%	Fail
80%	50	1.5%	Fail

% Acetonitrile + DMF	PLGA mg/ mL	% PVP (non-solvent)	Result
80%	50	2.5%	Fail
80%	50	2%	Fail
80%	50	1.5%	Fail

These particles were characterized to assess size and shape in order to determine if this method generated the ideal particle that could penetrate the airway mucus. A particle size of 200 nm is considered be the optimal nanoparticle size.[4] Particle sizes in the presence and absence of drug (C58) were assessed (Figure 1). 5% C58-loaded particles resulted in larger particles of about 3500 nm. 1% C58-loaded particles did not efficiently load, but were of smaller size. Loading was demonstrated by light absorbance at 300 nm and 360 nm. 1% C58- loaded particles had an absorbance of 0.07 at 300 nm and 0.07 at 360 nm; and 5% C58-loaded particles had an absorbance of 0.302 at 300 nm and 0.132 at 360 nm. Thus, it can be concluded that particles loaded with 5% C58 could be formed by combining PLGA (50 mg/mL) with 3% PEG that was dissolved in 80% acetonitrile and dropped into 2% PVP.

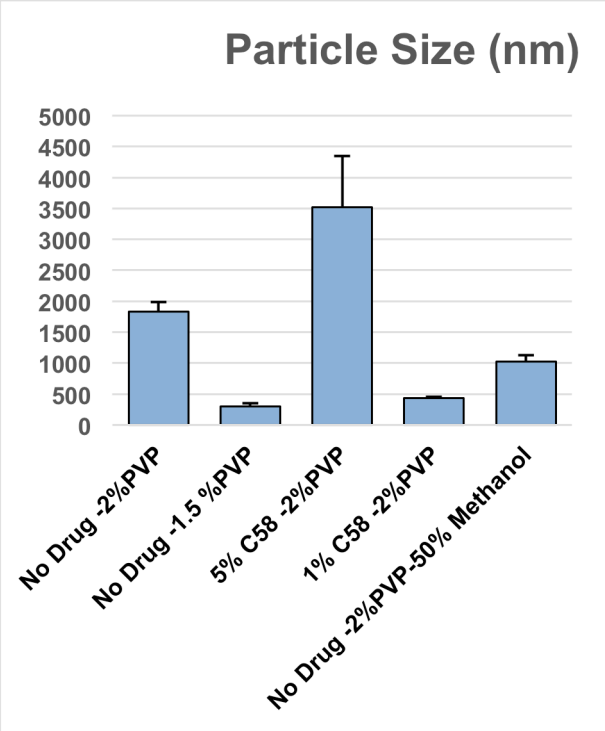

Figure 1. Define the size of C58 loaded particles

CHAPTER IV

CONCLUSION

PLGA-PEG nanoparticles can be fabricated using the nanoparticle precipitation method. Overall, it can be concluded that the concentration of PEG is critical for the manufacturing process. This conclusion is not surprising, as PEG is essential for the formation of the particle-solution interface. We found that 2.5% to 3% PEG was necessary, but higher concentrations may produce a higher particle yield with a smaller size. Further work will need to be performed in order to determine the optimal PEG concentration. If higher PEG concentrations are needed, the cost of particle generation will increase. Also, higher concentrations of PEG may trap C58 in the particle and not allow release into the mucous/biofilm.

Acetonitrile was an appropriate solvent at a concentration of 80%. Acetone or acetone plus DMSO are similar solvents, but did not result in particle formation. This result is a little disappointing, as acetone is considered a safer solvent. Thus, particles will need to be thoroughly washed before human usage.

Particles loaded with the antibiotic, C58, resulted in particle formation. Low concentrations of 1% C58 resulted in a particle size of 450 nm, which is close to the target particle size 250 nm, but they did not efficiently load. [4] However, nanoparticles loaded with 5% C58 resulted in large particles of 3500 nm. Smaller particle size and higher concentrations of antibiotics are optimal for penetration into the mucus and delivery of high concentrations of drug. Further experiments in which the PLGA concentration is reduced so that more C58 may be packed into the particle core may allow for the production of particles of smaller size. However, particles of 3500 nm may be workable. They will likely deposit in the small and medium size airways where the

infection occurs in CF patients. Indeed, in the classical study by Gebhart et at, particles of 3500 nm deposited at the appropriate site for the treatment of infection in the CF airway.[17] There is, however, a disadvantage, as particles of this size are removed from the airway via mucociliary clearance.[13] Nevertheless, the approach may be successful, because a recent publication showed that mucociliary clearance is reduced in patients with CF and absorbance from nanoparticles is enhanced.[18] Thus, the larger particles loaded with 5% C58 maybe an appropriate workhorse particle.

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